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# Tracing Dynamics and Clonal Heterogeneity of *Cbx7*-Induced Leukemic Stem Cells by Cellular Barcoding

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## SUMMARY

Accurate monitoring of tumor dynamics and leukemic stem cell (LSC) heterogeneity is important for the development of personalized cancer therapies. In this study, we experimentally induced distinct types of leukemia in mice by enforced expression of *Cbx7*. Simultaneous cellular barcoding allowed for thorough analysis of leukemias at the clonal level and revealed high and unpredictable tumor complexity. Multiple LSC clones with distinct leukemic properties coexisted. Some of these clones remained dormant but bore leukemic potential, as they progressed to full-blown leukemia after challenge. LSC clones could retain multilineage differentiation capacities, where one clone induced phenotypically distinct leukemias. Beyond a detailed insight into CBX7-driven leukemic biology, our model is of general relevance for the understanding of tumor dynamics and clonal evolution.

## INTRODUCTION

Leukemias are proliferative diseases that arise from HSCs or progenitors that fail to obey the regulatory signals that normally restrict their self-renewal and direct differentiation. Sequential “linear” acquisition of mutations in tumor suppressor genes or oncogenes has long been thought to drive leukemogenesis, as postulated in the original clonal evolution hypothesis (Knudson, 1971; Nordling, 1953; Nowell, 1976). However, the use of advanced genomic techniques to investigate clonal diversity and evolution now shows that tumors are often organized in a nonlinear, branching hierarchy (Anderson et al., 2011; Campbell et al., 2010; Jan and Majeti, 2013; Mullighan et al., 2008; Wu, 2012).

It had been shown that only a subfraction of cells within the leukemic cell population, termed leukemic stem cells (LSCs), possesses the ability to initiate and sustain disease (Bonnet and Dick, 1997; Lapidot et al., 1994). Early publications demonstrating heterogeneity within the LSC population utilized the detection of viral integration sites after transduction of acute myeloid leukemia (AML) cells and showed that human LSCs differ in their self-renewal capacities after transplantation in immunodeficient mice (Hope et al., 2004). However, it has recently been reported that xenotransplantation might not always reflect subclonal heterogeneity in patients’ leukemia due to species-specific selective pressures (Klco et al., 2014).

Later approaches employed fluorescence in situ hybridization (FISH) to monitor translocations and copy number alterations to investigate genetic heterogeneity within pediatric acute lymphoblastic leukemia (ALL) (Anderson et al., 2011). However, the resolution of this FISH-based

method to identify heterogeneity and subclonal origin is limited. Deep sequencing genomic DNA or RNA (exome sequencing) of malignant cells from patients provides the highest possible resolution for identification of mutations or other genetic abnormalities within a tumor. Such attempts have recently been made in leukemic patients (Landau et al., 2013; Sanders and Valk, 2013; Schuh et al., 2012). Although potentially very powerful, it is difficult to define (sub)clones based on a large set of genomic data from a pool of genetically diverse cells (Glauche et al., 2013), and it requires complex computational approaches and multiple assumptions. For example, the assumption that a certain mutation occurs only once and therefore represents a stable “unique mark” is often made. However, genomes of cancers are generally unstable, and the same mutation may have occurred twice. Another assumption is that the allelic frequency with which a particular mutation occurs depends on the time point of its origin. However, this does not always have to be the case, as primitive LSCs might actually be dormant. In addition, it remains difficult to distinguish which genetic abnormalities are causal to disease progression and which are functionally neutral passenger mutations (Welch et al., 2012).

Although previous studies begin to recognize the potential complex genetic architecture of leukemia, unambiguous longitudinal detection of leukemic clones remains difficult to achieve. Ideally, clones should be prospectively defined by unique labeling of cells before tumor initiation, and detection of different clones should be performed at high resolution. A recently described method, which would potentially accomplish this, is the marking of cells by the introduction of a unique, heritable mark that can



be detected in its offspring experimentally (Bystrykh et al., 2012; Gerrits et al., 2010; Naik et al., 2014; Schepers et al., 2008). These methods rely on the viral integration of a random “barcode” sequence of fixed length in the genome of target cells. After transplantation of barcoded cells, their offspring can be traced by quantifying the abundance of unique barcodes using deep sequencing.

Previously, we have shown that overexpression of the Polycomb PRC1 member *Cbx7* in bone marrow cells causes leukemia (Klaue et al., 2013). While typically a single oncogene causes one specific tumor type, the epigenetic modifier CBX7 causes a wide spectrum of leukemias, including T-ALL, erythroid, and undifferentiated leukemias. Since only long-term hematopoietic stem cells (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs), but not lineage-restricted progenitors are responsive to *Cbx7* overexpression (Klaue et al., 2013), the different types of leukemias are not likely to depend on the cell of origin in which *Cbx7* is overexpressed. Rather, the phenotypic variation seems to be an inherent virtue of CBX7.

In the present paper, we have generated a mouse model in which overexpression of *Cbx7* serves as the initial leukemic “hit” and every pre-LSC is uniquely labeled by a barcode. We show how our approach allows for the identification of LSC-derived clones in the transplanted primary and secondary recipients. We prospectively describe clonal dynamics in mice that succumb to leukemia and highlight the complexity of clonal evolution.

## RESULTS

### Overexpression of *Cbx7* in Primitive Bone Marrow Cells Induces Distinct Types of Leukemia

We previously reported that CBX7 has a strong, but dynamic oncogenic potential (Klaue et al., 2013). Overexpression of this Polycomb gene in hematopoietic stem and progenitor cells (HSPCs) induces multiple leukemia subtypes (Figure 1A) (Klaue et al., 2013). Morphological and immunophenotypic analyses (Figure 1; Table S1 available online) of cells isolated from various hematopoietic tissues such as blood, bone marrow, spleen, and lymph nodes showed that the majority of mice developed a T cell leukemia. Some mice developed an erythroid leukemia, and undifferentiated (lineage negative) leukemias were also detected (Figure 1A) (Klaue et al., 2013). Typically, mice were anemic and spleens were profoundly enlarged, while white blood cell counts in peripheral blood were increased in most mice (Figure 1B; Table S1).

The *Cbx7*-induced leukemic mice that were reported in this previous study were established by transplantation of HSPCs transduced with *Cbx7* barcode vector libraries, composed of 200–300 unique barcodes (Figure 1C). This al-

lows for the sensitive identification of single LSC-derived clones in the transplanted recipient. Clonal waves of normal and LSC contributions to the blood and emergence and persistence of clonal dominance were analyzed by regular blood sampling (Figure 1C). The additional clonal compositions in bone marrow and spleen were analyzed postmortem, after leukemia development. In multiple instances, bone marrow cells were serially transplanted in secondary and tertiary recipients (Figure 1C). Altogether, this experimental design allowed us to precisely determine the relative contribution of distinct clones to leukemia initiation and progression.

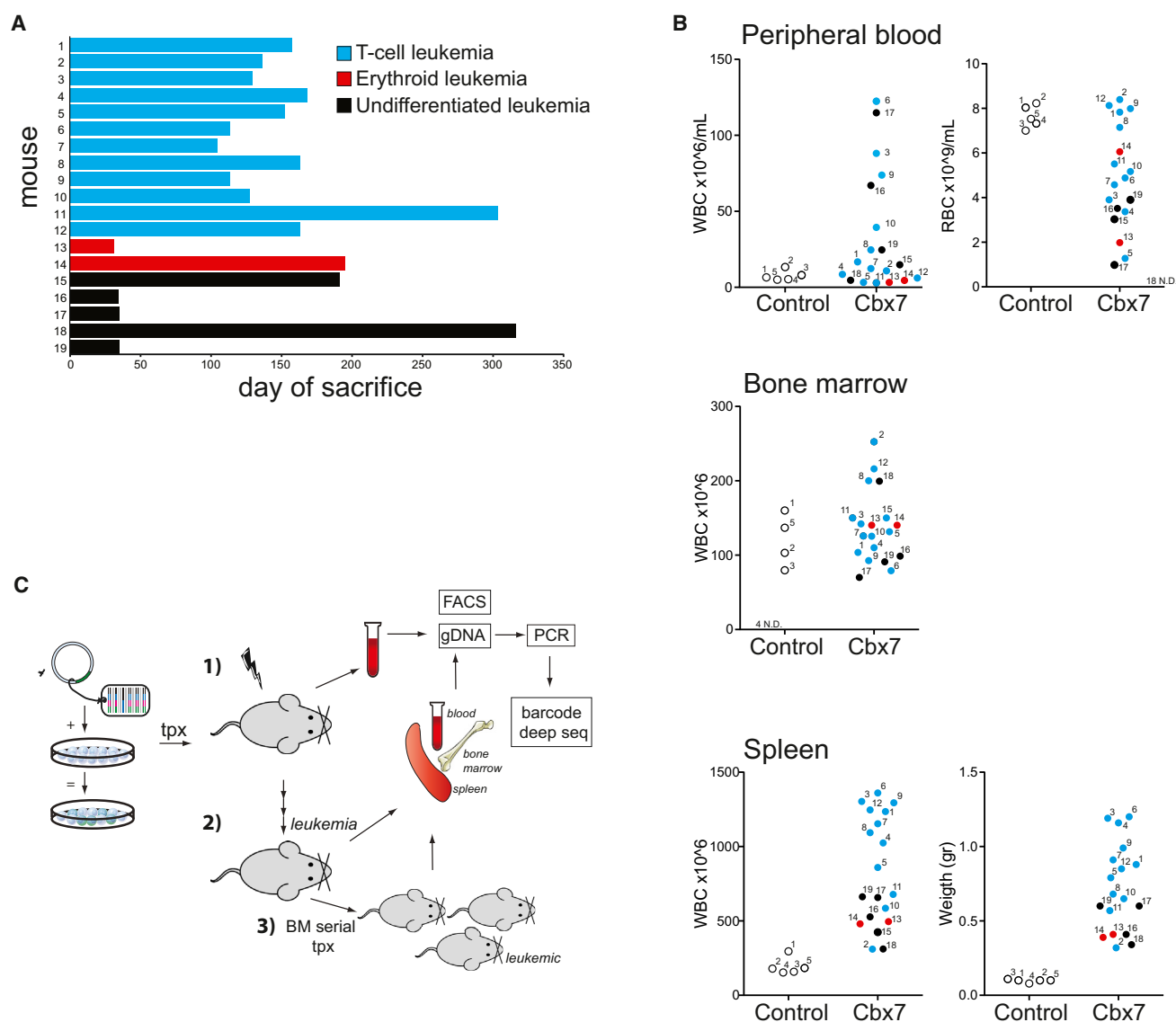
### *Cbx7*-Induced Leukemias Are Monoclonal or Oligoclonal

We analyzed contributions of major and minor clones in different hematopoietic tissues in every individual control or leukemic mouse and retrieved barcodes (Table S2) from blood, bone marrow, and spleen samples taken at the time of sacrifice (Figures 1A–1C).

Occasionally, multiple vectors carrying different barcodes may have integrated in a single cell. In these instances, a LSC clone can be composed of multiple barcodes. We indeed found barcodes that were likely to belong to the same clone since the highly coordinated behavior of such barcodes was unlikely to occur by chance (Bray-Curtis distance, see Supplemental Experimental Procedures). In the figures we have indicated these linked barcodes by the suffix a, b, c (for example, in mouse 9 barcode 1a–1c represent clone 1, and barcode 2a and 2b represent clone 2; Figure 2C). These mice were transplanted with bone marrow cells where 40% transduction efficiency was reached. Since the chance of the occurrence of three to four integration sites under these conditions is small (expected ~1%–2% of cells with three and 0%–16% with four integrations (Poisson,  $p < 0.05$ ), this indicates that high *Cbx7* gene dosage due to multiple vector integrations might have a positive effect on cell proliferation and clonal selection.

As expected, bone marrow cells transduced with barcoded retroviral control vectors repopulated all hematopoietic lineages (Figures 2A and 2B). The hematopoietic system of most of these control mice was repopulated by 13 to 26 different clones (Figure 2B, mice 1 and 2; Figure S1 mice 4 and 5). Typically, two to three major clones were found to predominate in the various tissues (blood, bone marrow, and spleen) and made up approximately 30%–60% of all hematopoietic cells. One mouse displayed a biclonal hematopoietic system (in blood, bone marrow, and spleen) (Figure 2B, mouse 3), yet this did not evolve in malignancy.

In Figures 2C–2E, we display lineage contributions and the clonal composition of various hematopoietic tissues (blood, bone marrow, and spleen) of individual leukemic



### Figure 1. Cbx7-Induced Leukemia Phenotypes

(A) HSPCs were transduced with a barcoded *Cbx7* vector library and transplanted in 19 irradiated recipients (Klauke et al., 2013). Mice developed different types of leukemia indicated by the color of the bar, at indicated time points. The number of each bar reflects to the unique mouse identifier number that is used throughout this manuscript.

(B) Leukemic mice show increased white blood cell counts in the blood, anemia, variable bone marrow cellularity, and increased spleen size and cell numbers herein. Also see Table S1.

(C) Overview of the experiments. Clonal contributions of HSCs to the blood were analyzed by regular blood sampling (weeks 4, 8, 16, and 24). Mice were sacrificed when leukemia developed, and the clonal composition in blood, bone marrow, and spleen was subsequently analyzed. Bone marrow cells were isolated from primary leukemic mice and serially transplanted in secondary recipients. For clonal analysis, cells were analyzed and/or purified by flowcytometry, and barcodes were retrieved from gDNA using deep sequencing.

mice. As we observed a high and unpredictable variability in clonal composition of leukemias, we below describe most of our findings case by case.

In general, the hematopoietic system of leukemic mice (Figures 2C–2E, S2A, and S2B) was composed of fewer clones compared with healthy controls (Figure 2B; Fig-

ure S1). However, in many cases, the leukemic compartment of T cell leukemic mice did not show monoclonality (Figures 2C and S2A), with the exception of two T cell leukemias (Figure 2C, mouse 1; Figure S2A, mouse 6). In these oligoclonal leukemias, two to four LSC clones simultaneously contributed to malignant outgrowth of T cells in



the blood, bone marrow, and spleen, resulting in increased white blood cell counts and splenomegaly. As an example, mice 4 and 9 (Figure 2C) both showed an oligoclonal T cell leukemia. In mouse 4, the expansion of malignant CD3 $\epsilon^+$  cells was most profound in the spleen. In this mouse, clones 2 and 3 together constitute ~85% of cells in the spleen, and the same clones are predominant in fluorescence-activated cell sorting (FACS)-purified CD3 $\epsilon^+$  cells (Figure 2F). In mouse 9, two clones (clones 1 and 2) are responsible for the expansion of malignant CD3 $\epsilon^+$  cells (Figures 2C and 2F). In this mouse, but also in mouse 5 and 11 (Figure S2A), malignant clones predominated the blood and spleen, but not the bone marrow, in which no overt signs of leukemia were detected. This suggests migration of malignant LSCs and/or their progeny to the blood and spleen, after which relatively normal hematopoiesis persisted in the bone marrow by the activity of other clones.

Mouse 3 (Figure 2C) presented with a large number of CD3 $\epsilon^+$  T cells, but also the number of TER119 $^+$  erythroid precursors and immature hematopoietic cells (LIN $^-$ ) was excessive (see Figure 1B for absolute cell counts). Three distinct clones (clone 1, 2, and 3) were likely collectively responsible for this malignancy. There are two explanations for such oligoclonal contributions to phenotypically distinct cell types. First, primitive LSCs may have retained the ability to (an extent of) multilineage differentiation. Alternatively, distinct LSC clones may have contributed to the expansion of separate lineages. We cannot formally discriminate between these possibilities since not all distinct cell populations have been separately analyzed for their clonal composition. However, since all three LSC clones contributed to malignant CD3 $\epsilon^+$  T cells (Figure 2F) and since the prevalence of these clones together is ~95% in the spleen, these very same clones most likely also contributed to the expansion of erythroid and immature cells. This suggests multilineage differentiation capacities of LSCs. Additional data that are described below further attest to this notion.

The hematopoietic system of the two mice that developed an erythroid leukemia (mice 13 and 14, Figure 2D) showed oligoclonality in blood, bone marrow, and spleen. In contrast, the hematopoietic system of mice that developed an immature leukemia was always found to be monoclonal (Figures 2E and S2B).

We also analyzed the clonal composition in purified hematopoietic cell populations of individual mice. To this end, different cell lineages from the blood and spleen were FACS sorted, and barcodes were retrieved from genomic DNA using deep sequencing (Figures 2F and S2C). We found that minor, nonmalignant clones could still contribute to residual normal myelopoiesis (GR1 $^+$  cells) in leukemic mice. In addition, dominant barcodes could mark both malignant T cells as well as nonmalignant gran-

ulocytes (Figures 2F and S2C). Thus, the cell from which the leukemia derived possessed both lymphoid and myeloid differentiation capacities, which suggests that the oncogenic origin of leukemias in this mouse model lies within multipotent stem cells.

### The Onset of Clonal Dominance

We next assessed the emergence and kinetics of clonal dominance during leukemia development.

At early time points after transplantation, only a few (two to eight) clones contributed to blood cell regeneration (Figures 3 and S3). Some clones that later became highly malignant contributed to normal hematopoietic reconstitution and showed multilineage differentiation capacities early after transplantation (Figure 3, for example, mouse 1 clone 1, mouse 15 clone 1). At the time point of leukemia development, these initially multilineage clones showed malignant proliferation in preferentially one hematopoietic cell type.

We also found cases where the minor clones remained undetectable in the blood at earlier time points and only later became dominant and contributed to leukemia development (Figure 3, mouse 5 clone 6, mouse 18 clone 1; Figure S3, mouse 11 clone 1).

Taken together, we show that the emergence of malignant cells does not always coincide with the expansion of a single malignant clone. Premalignant clones can contribute to multiple lineages at earlier time points, and dormant clones can suddenly emerge and become malignant. Therefore, our data reveal a highly variable and unpredictable timing of the onset of clonal dominance.

### Clonal Stability after Serial Transplantation

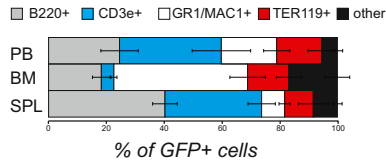
Next, we tested whether leukemias were transplantable and whether the leukemic subtypes, as assessed by organ involvement, tissue morphology, and immune phenotype, were maintained from the original donor to the recipients. For this purpose, we serially transplanted bone marrow cells from leukemic mice into secondary recipients. Once more, it appeared that leukemias behaved unpredictably when challenged by serial transplantation. For that matter, we describe these serial transplantation studies case by case.

#### Heritability of Clonal Dominance and Leukemia Phenotype

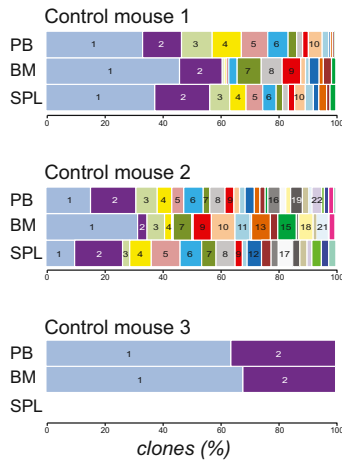
In the first serial transplantation experiment (Figure 4A), we observed a very stable pattern of both disease phenotype and clonal dominance. Three secondary recipient mice transplanted with bone marrow cells from a primary recipient with a monoclonal T cell leukemia all developed T cell leukemias as well (Figures 4B and 4C; also see Table S1). Barcode analyses revealed that the disease-causing clone was identical in the donor mouse and all individual recipients (Figure 4D, mouse 1 donor, and recipients 1-1, 1-2, 1-3, clone 1).



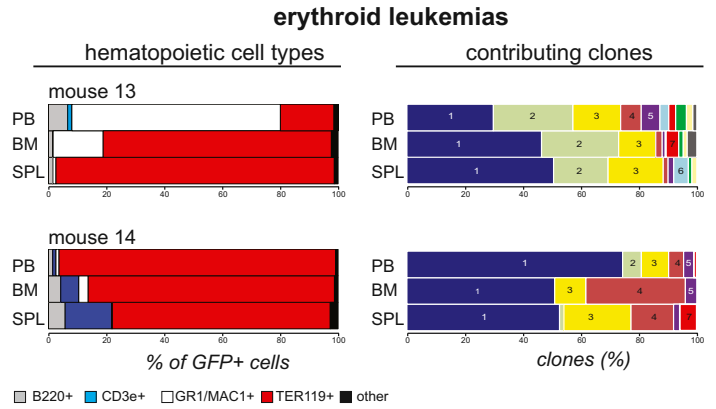
A



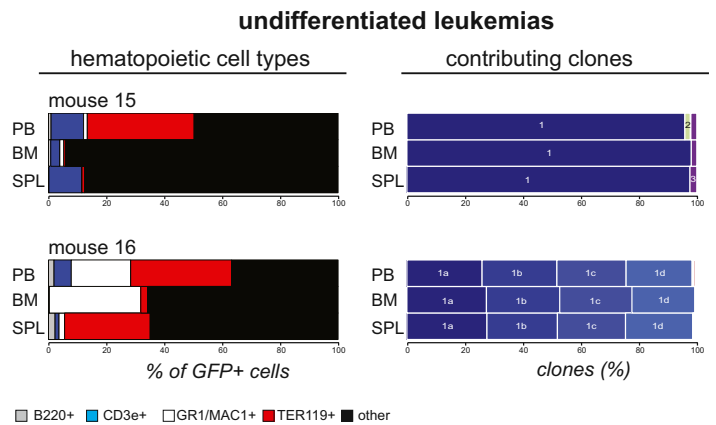
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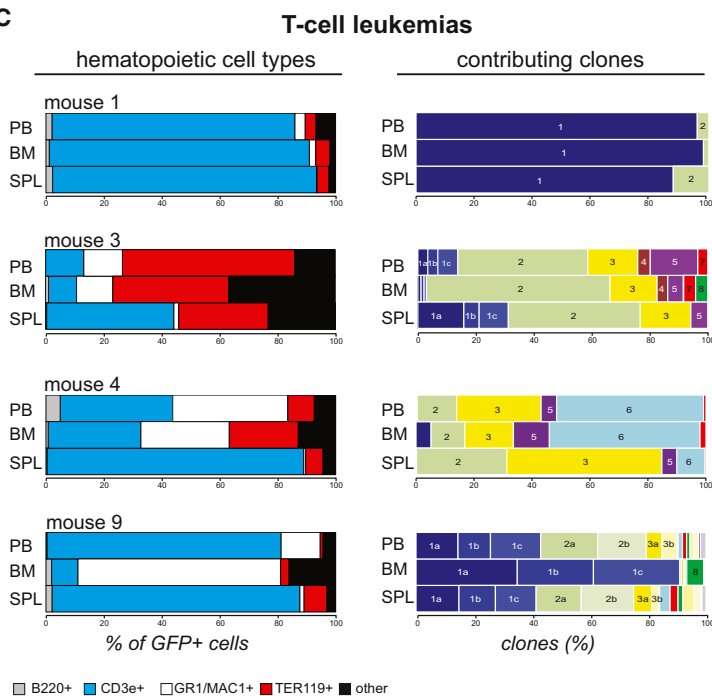
D



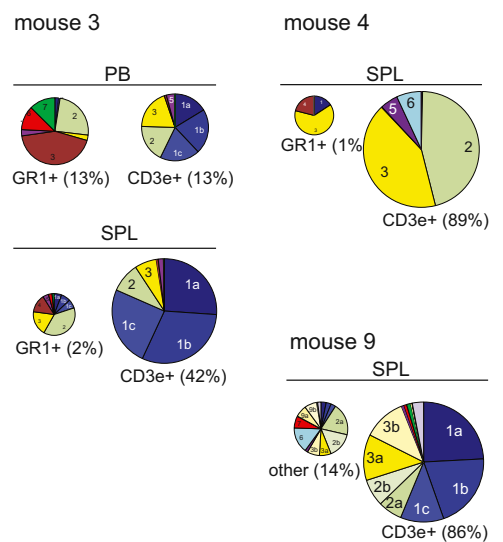
E



C



F



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### Clonal Instability after Serial Transplantation

Although the behavior of some clones remained unaltered, we frequently observed the appearance of different leukemic phenotypes after serial transplantation (Figures 5, 6, S4, S5A, and S7). Immature (Figures 5A–5C) and erythroid leukemias (Figures 6A–6C and S7A–S7C) appeared after serial transplantation of bone marrow cells from T cell leukemic mice. In these cases, malignant cells from the recipient mouse displayed different cell-surface markers [for example, lineage negative (Figures 5A and 5C, mouse 4-3) or TER119<sup>+</sup> (Figures 6A and 6C, mouse 5-5; Figures S7A and S7C, mouse 2-3)] instead of CD3e<sup>+</sup>, which was expressed on the leukemic cells from the donor (also see Table S1). In both these cases, lymph nodes were not enlarged, while this is typical for T cell leukemias (Figure S4).

To test whether these secondary leukemias were correctly identified as a different subtype, we assessed genome-wide gene expression from spleen cells from six T cell leukemic mice, five erythroid mice, and one mouse that developed an immature leukemia (Figures 5E, 6E, 7E, and S5B).

Three hundred forty-three genes showed significantly higher expression in T cell leukemias compared with erythroid leukemias, while conversely, 225 genes were significantly upregulated in erythroid leukemias as compared with T cell leukemias (Benjamini Hochberg,  $p < 0.01$ ; Figure S5B and Table S3). Complete linkage hierarchical clustering on the basis of these differentially regulated genes showed that samples clustered by leukemia subtype and not by transplantation experiment (Figure S5B). Genes that showed a higher expression in leukemic T cells were overrepresented in pathways involved in normal T cell differentiation and lymphocyte activation (Figure S5C, GO-analysis false discovery rate [FDR]  $< 0.05 \times 10^{-3}$ ), while genes that were high expressed in erythroid leukemias were enriched in pathways associated with hemoglobin biosynthesis (Figure S5D).

Next, we compared gene expression profiles of leukemic T cells and leukemic erythroid cells with previously published data sets (GSE6506, Chambers et al., 2007, and GSE18669, Weishaupt et al., 2010) in which different normal hematopoietic cell types had been profiled. We extracted differentially regulated genes between normal hematopoietic subsets and compared this with differentially expressed genes between T cell leukemic and erythroid leukemic cells. Genes found upregulated in leukemic T cells were specific for normal T cell subsets (hypergeometric test,  $p < 0.05$ ; Figure S5E), while genes differentially upregulated in leukemic erythroid cells were specific for normal erythroid cells or erythroid precursor cells (hypergeometric test,  $p < 0.05$ ; Figure S5F).

Together, these data show that secondary leukemias can be phenotypically and functionally distinct from the disease as it occurred in the original donor. Different scenarios of clonal evolution explain these lineage conversions, as is described in detail below.

**Activation of Dormant LCS Clones.** To monitor the clonal dynamics associated with the appearance of different leukemic phenotypes after serial transplantation, the contribution of each clone to leukemia progression in secondary recipient mice was determined.

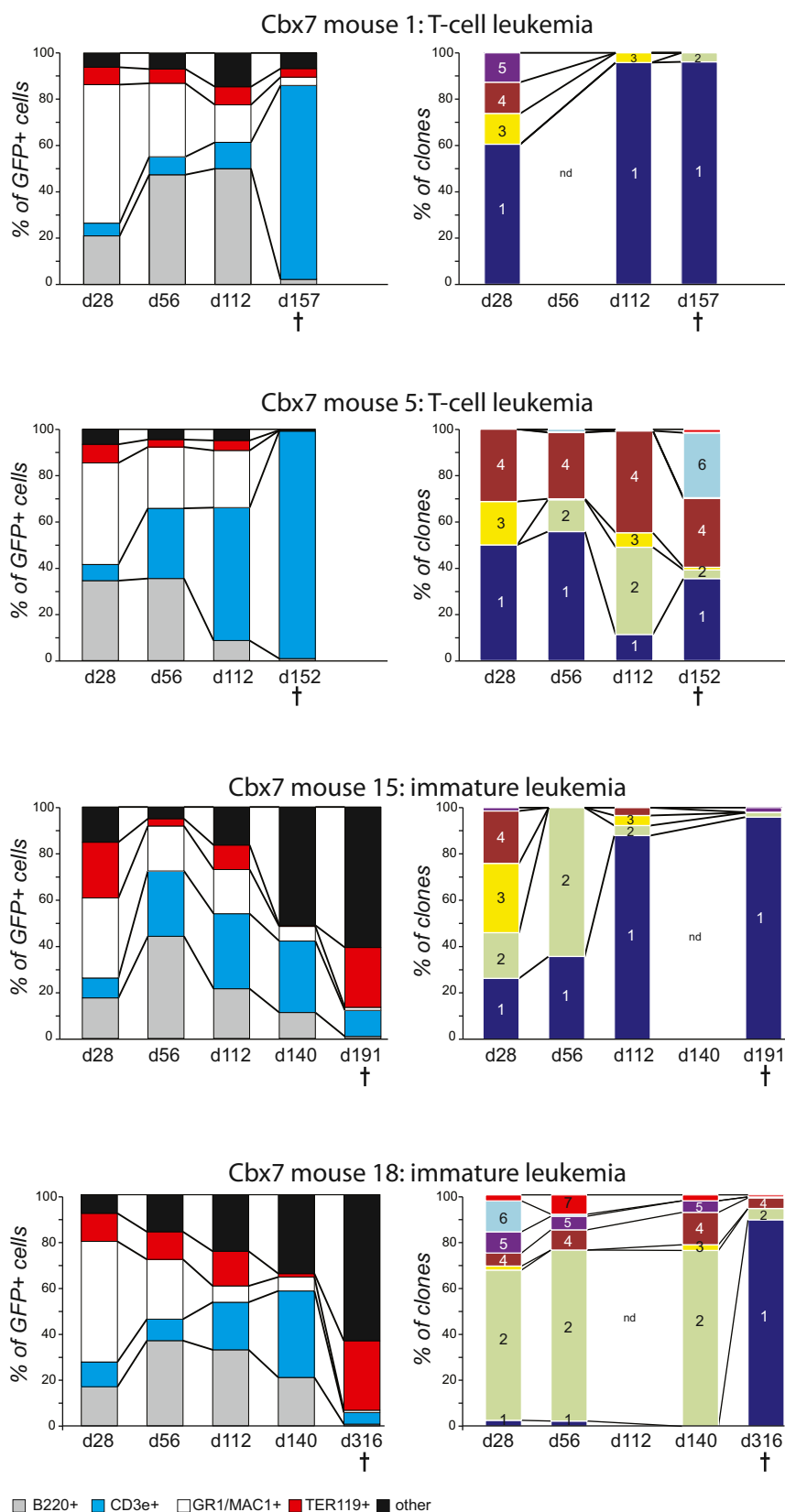
Bone marrow cells from donor mouse 4, with an oligoclonal T cell leukemia, were serially transplanted in three recipient mice, of which recipient 4-1 and recipient 4-2 also developed a T cell leukemia (Figures 5A–5C and 5E). In contrast, recipient 4-3 developed an immature leukemia.

We observed that the appearance of a different leukemia subtype after serial transplantation coincided with the emergence of a new dominant clone (Figure 5D). Different cell populations were FACS purified from the blood and spleen of secondary recipients, and the contribution of each clone to different cell lineages was determined. Clones 2 and 3 were identified as the malignant clones present in the donor mouse since these cells contributed

### Figure 2. Clonality in Control and Cbx7-Induced Leukemic Mice

(A) Percentage of different GFP<sup>+</sup> cell types in blood, bone marrow, and spleen of control mice ( $n = 5$ , mean  $\pm$  SD).  
 (B) The number of clones and their relative contribution to blood, bone marrow and spleen is indicated for three mice that were transplanted with barcoded control vector transduced cells (also see Figure S1 for more mice).  
 (C–E) Mice were transplanted with bone marrow cells transduced with barcoded Cbx7-expression vectors. The contribution of transplanted GFP<sup>+</sup> cells to the different cell lineages in blood, bone marrow, and spleen is shown in all panels on the left. The corresponding contribution of different clones to the blood, bone marrow, and spleen is shown in panels on the right in T cell leukemic mice (C; also see Figure S2A), erythroid leukemic mice (D), and mice with undifferentiated leukemias (E; also see Figure S2B).  
 (F) Multiple FACS-purified hematopoietic lineages from the blood and/or spleen of mice 3, 4, and 9 were clonally analyzed in detail. Shared but also unique barcodes contribute to different cell lineages. The size of the pie reflects the clonal contribution of cells in the indicated tissue. Also see Figure S2C.

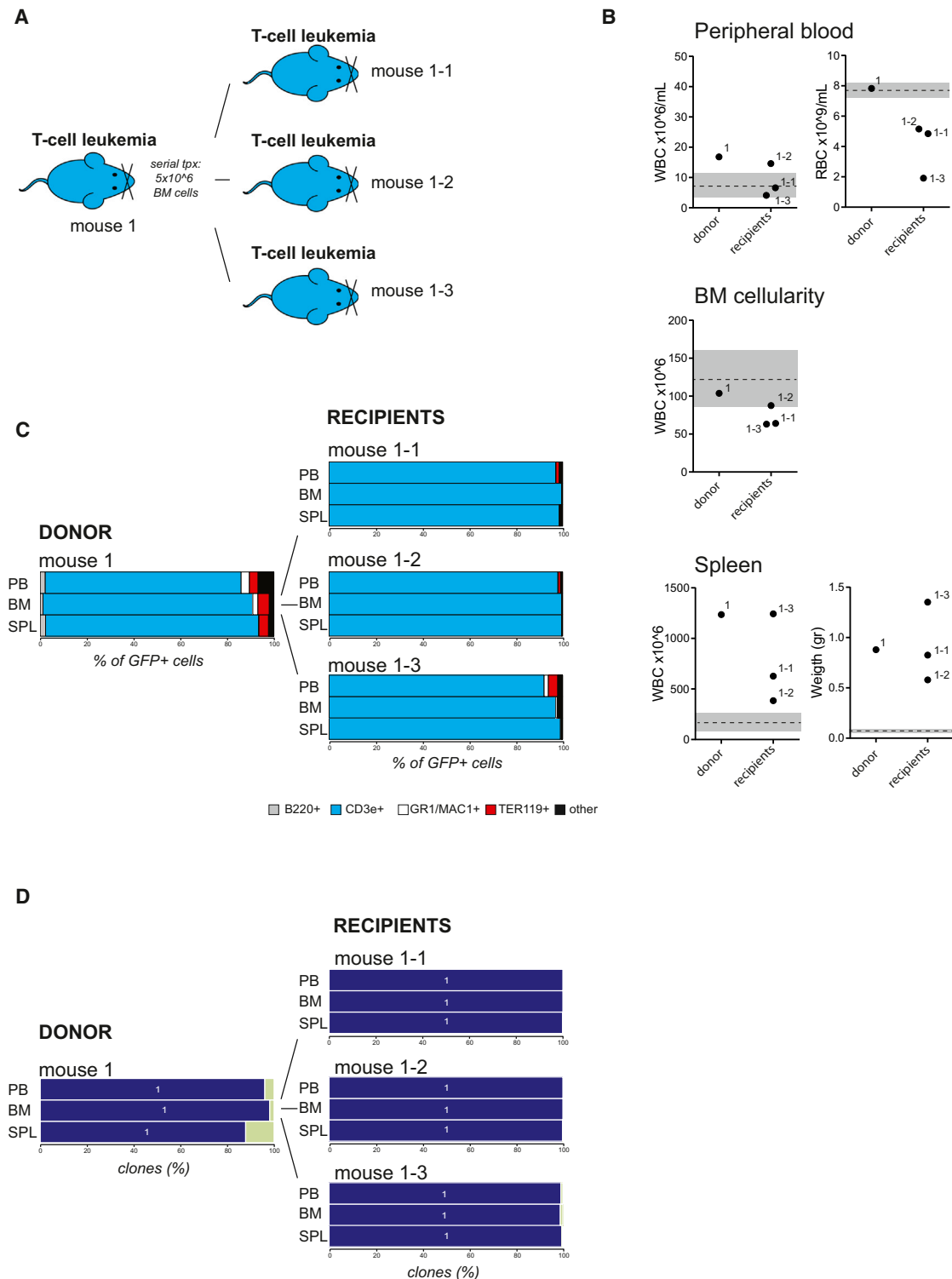
For all individual mice, different clones are indicated by different colors and are uniquely numbered. The barcode that was most frequently found among all samples from one transplantation experiment was numbered barcode 1 (blue), and the second most frequently appearing barcode was numbered barcode 2 (green) etc. In cases where multiple barcodes integrated in a single cell, barcodes were numbered 1a, 1b, 1c, etc.



**Figure 3. Onset of Clonal Dominance in Cbx7-Induced Leukemic Mice**

The left panels show the percentage of multiple GFP+ donor-derived hematopoietic cell types in blood on indicated time points, as analyzed by FACS in four mice. Panels on the right display the contribution of different clones to the blood on indicated time points. The final time point is the day of sacrifice (†) at clear signs of morbidity. See Figure S3 for additional data on more mice.





**Figure 4. Heritability of Clonal Dominance and Disease Phenotype**

(A) Experimental setup. Serial transplantation of bone marrow cells isolated from mouse 1 with a T cell leukemia into three secondary recipients (1-1, 1-2, and 1-3), which all developed T cell leukemias.

(B) Cell counts in blood, bone marrow, and spleen and spleen weight of all mice are indicated. Reference blood counts of control nonleukemic mice are indicated by the gray box (dashed line refers to the mean values).

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to the expansion of CD3 $\epsilon$ <sup>+</sup> cells primarily in the spleen (Figure 5C). The same two clones were also highly dominant in expanded CD3 $\epsilon$ <sup>+</sup> cells in blood (68% and 95% of cells) and spleen (91% and 95% of total cells) from recipients 4-1 and 4-2 that developed T cell leukemias, similar to the donor. However, the immature leukemia in recipient 4-3 was of a different clonal origin. Different clones (clone 1 and clone 4) were responsible for the expansion of immature cells, which composed 96% of cells in the blood and 98% of cells in the spleen. Interestingly, clone 1 and clone 4 also contributed to a modest expansion of immature cells in the spleen of recipient 4-1 (30% of total cells). These clones were barely detectable in the hematopoietic system of the donor mouse at leukemia diagnosis and thus must previously have been relatively dormant.

**Multilineage Differentiation Capacities of LSC Clones.** A similar pattern of activation of minor clones was observed in the third serial transplantation experiment (Figure 6). While in donor mouse 5 clone 1 drove T cell malignancy predominantly in the blood and spleen (Figures 6A–6C), in recipient mice, different clones were contributing to leukemia (Figures 6D and 6F). Only recipient 5-2 inherited the same dominant clone (clone 1) as one of the disease-causing clones in the donor, driving a similar disease phenotype (T-ALL). Clone 3, which was relatively minor in the donor mouse, became highly dominant after serial transplantation, causing T cell leukemias in recipients 5-1 and 5-3. Although clone 2 (green) was highly prevalent in the bone marrow of the donor mouse, at the time of sacrifice, it was not yet malignant since it did not contribute to the expansion of T cells in blood and spleen. However, this clone developed into a T cell leukemia in recipient 5-4.

Surprisingly, the very same clone caused development of an erythroid leukemia in recipient 5-5, as shown by organ morphology (Figure S4), FACS phenotyping (Figure 6C), and gene expression (Figures 6E and S5). We verified the clonal identity of these samples by Sanger-sequencing (data not shown) and by integration site analysis using inverse PCR (Figure 6F). We found the same vector integration site (into chromosome 4) in bone marrow cells from both mouse 5-4 and mouse 5-5 and validated by conventional PCR and Sanger sequencing that this integration corresponds to clone 2. Mice carrying dominant clones do not show abnormal expression of genes within a 50 kb distance of the vector integration site (Figures S6A–S6C), suggesting that insertional mutagenesis did not play any detectable role in leukemia initiation and progression in our experiments.

The next experiment resulted in similar observations (Figure S7). Serial transplantation of bone marrow cells from a mouse with a T cell leukemia (mouse 2) also resulted in development of erythroid leukemia in one recipient (mouse 2-3, Figures S7A–7C, S7E, and S5). Tertiary transplantation of bone marrow cells of this recipient with erythroid leukemia resulted in stability of disease phenotype, as all four tertiary recipients also developed erythroid leukemias.

While in the original donor clone 2 induced leukemia and contributed to malignant T cells, clone 1 became dominant after serial transplantation in recipients 2-1 and 2-3 (Figure S7D). While in recipient 2-1 this clone caused a T cell leukemia, in recipient 2-3 and its tertiary recipients (mouse 2-3-1, 2-3-2, 2-3-3, and 2-3-4) it caused development of erythroid leukemias (Figure S7D).

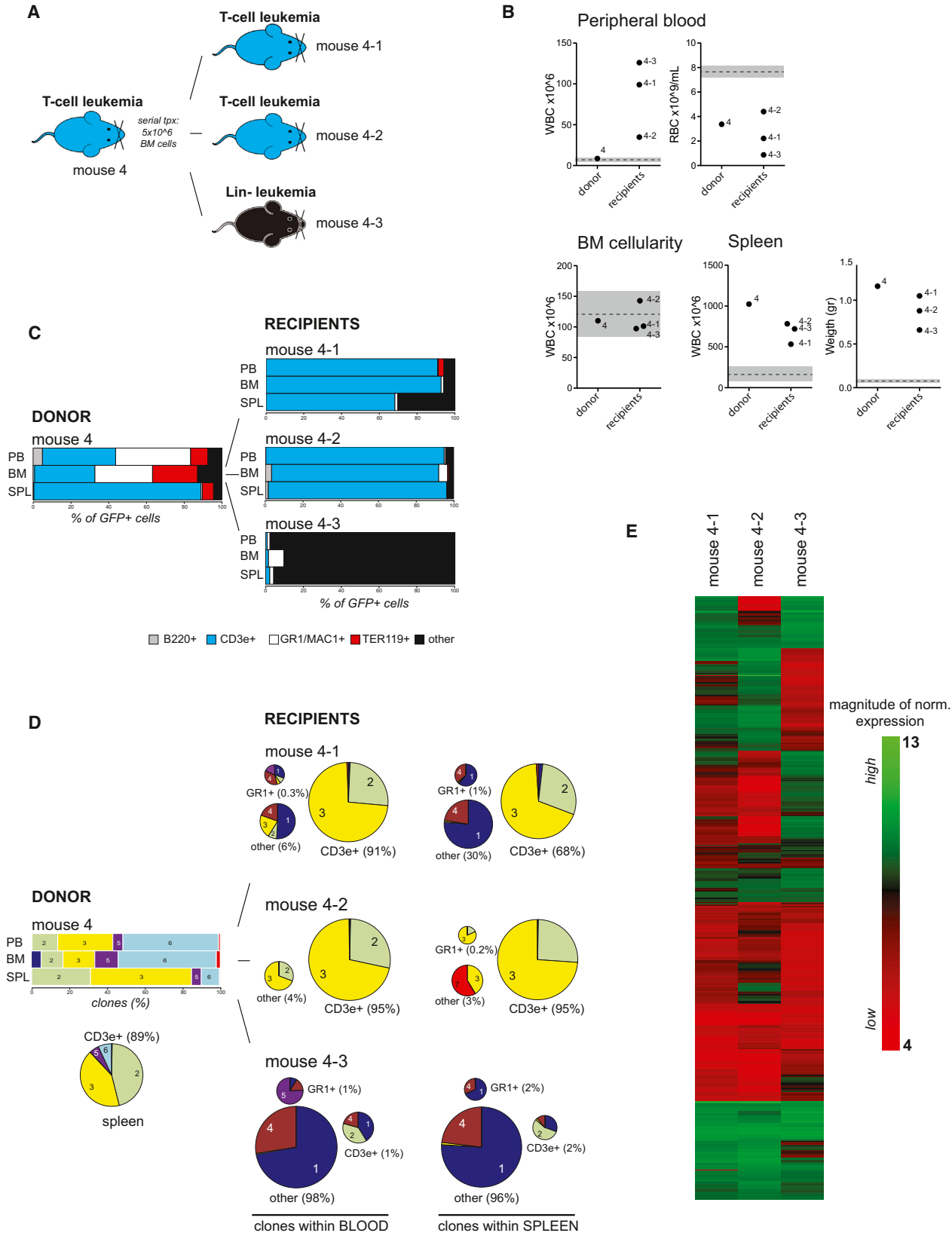
This observation in two independent transplantation experiments shows that a single clone is able to induce two morphologically, phenotypically immune, and functionally distinct types of leukemia.

**Differentiation of LSC Clones after Serial Transplantation.** Finally, we observed cases of evolution of a leukemic clone from an undifferentiated phenotype to a differentiated lymphoid lineage. Bone marrow cells from a mouse with an immature leukemia, which did not express any of the immunophenotypic lineage markers, were serially transplanted into three recipient mice (mouse 15, Figure 7A). Strikingly, all three recipients developed a CD3 $\epsilon$ <sup>+</sup> T cell leukemia (Figures 7B and 7C; Table S1). A single clone (clone 1) was highly dominant in the donor as well as in all recipients (Figure 7D). To verify whether these similar barcodes truly belong to the very same clone, clonal identity was validated by integration site analysis (integration into chromosome 1, data not shown). Thus, T cell leukemias in the recipients originate from the same LSC clone as the undifferentiated leukemia in the donor. Most likely, differentiation of a primitive LSC clone into the lymphoid lineage after serial transplantation resulted in a phenotypically distinct leukemia type in all recipients.

Altogether, these observations show that multiple clones with distinct leukemic properties can coexist in a single mouse. Minor LSC clones with similar or different differentiation potential can be relatively dormant in the bone marrow of a leukemic mouse and progress to full-blown leukemia only after serial transplantation. In addition, some LSC clones retain multilineage differentiation capacities, and one LSC clone can therefore induce phenotypically distinct leukemias.

(C) Percentage of different donor-derived GFP<sup>+</sup> hematopoietic cell types in blood, bone marrow, and spleen of mouse 1 and its recipients.

(D) The contribution of different clones to the blood, bone marrow, and spleen of mouse 1 and its recipients. Different colors represent different clones and are indicated by different numbers.



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## DISCUSSION

In early tumor models, malignant cells were thought to descend from a single common ancestor whose offspring sequentially acquired multiple mutations or other genetic abnormalities in time (Knudson, 1971; Nordling, 1953; Nowell, 1976). This implied that all cells in a full-blown tumor were genetically and functionally homogenous. However, emerging evidence suggests that leukemias (and other tumors for that matter) are comprised of a multitude of different subclones resulting in heterogeneity (Anderson et al., 2011; Landau et al., 2013; Magrangeas et al., 2013; Mullighan et al., 2008; Notta et al., 2011; Sanders and Valk, 2013; Schuh et al., 2012; Welch et al., 2012). This concept has thus far only been partially confirmed since previous experiments did not uniquely mark individual cells to allow prospective identification of leukemic clones. In the current study, we used a barcoding tool combined with overexpression of *Cbx7*, an epigenetic modifier that we recently found to regulate self-renewal of HSCs (Klauke et al., 2013), to discern patterns of clonality in highly variable leukemic subtypes. We delineate leukemic heterogeneity with high resolution and document the coexistence of (sometimes quiescent) LSCs with different leukemic properties in a single tumor.

Our analysis revealed three general patterns of clonal evolution. First, some leukemic clones are highly stable and dominant. These clones rapidly and strongly dominate the hematopoietic system of the primary donor mouse and upon serial transplantation cause a similar type of leukemia in secondary recipients. A second pattern consists of minor clones in primary donors that become activated and highly dominant only after serial transplantation. Leukemias in these recipient mice are thus of different clonal origin than the disease-causing dominant clone in the original donor mouse. As a consequence, the leukemia in the recipients can be either similar or different as the one observed in the primary donor. Third, we observed evolution (or differentiation) of leukemic clones. In these cases, the leuke-

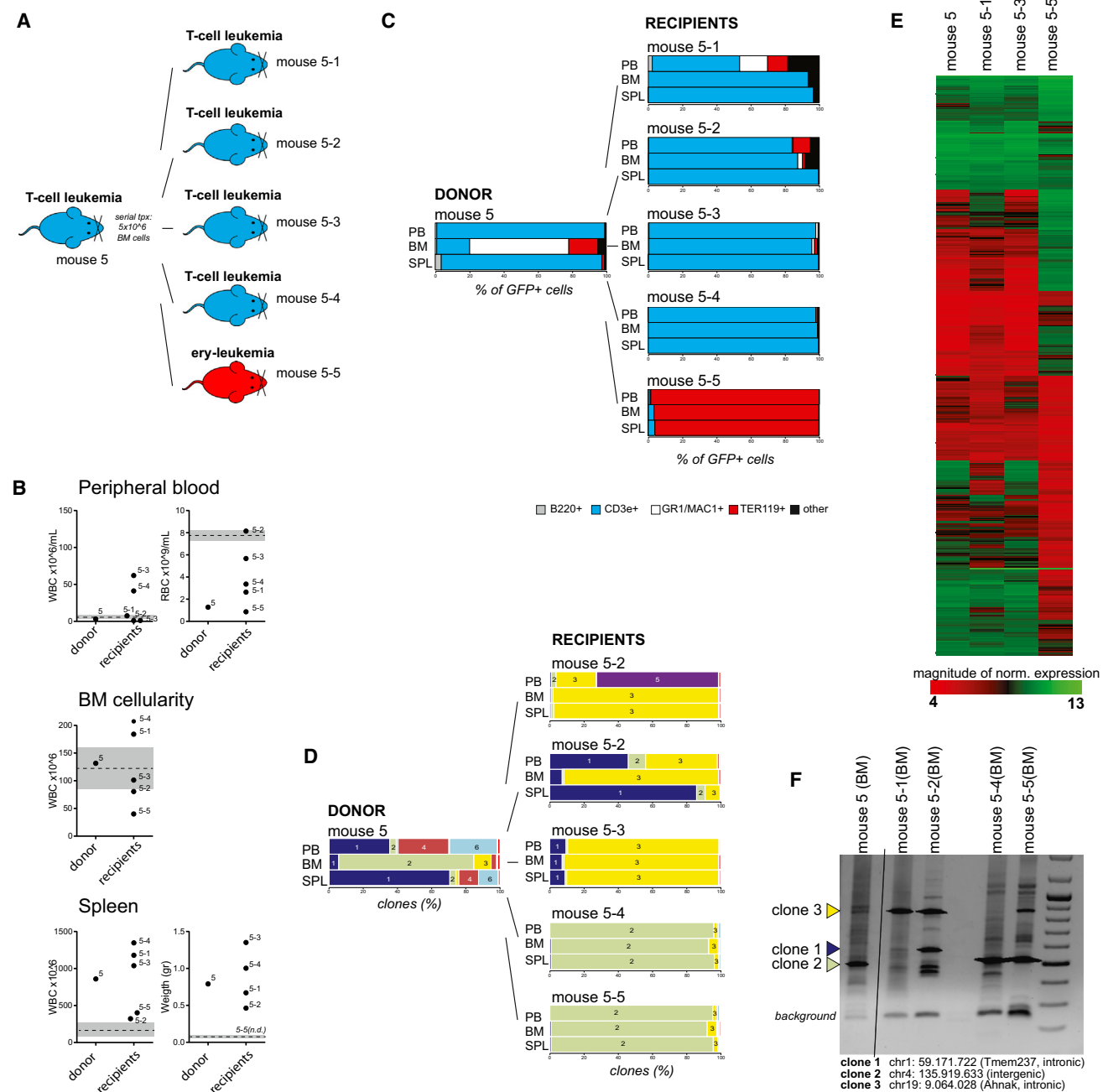
mias in the donor and the recipients are of the same clonal origin, but in the recipients, the leukemia manifests as a different clinical subtype. In all three cases we show that clonal dominance is not a prerequisite for end-stage leukemia. Some diseased mice displayed an oligoclonal leukemia, but in addition, minor clones can be present in the bone marrow of a leukemic mouse and progress to leukemia only after serial transplantation.

Additional genetic or epigenetic abnormalities that occur after the first leukemia-predisposing event (high *Cbx7* expression in primitive cells) within a subset of cells from one leukemic clone (cells that carry the same barcode) are likely to be the driving force behind the leukemia dynamics that we observed in our study. Since CBX7 is an epigenetic modifier, it is tempting to speculate that overexpression of *Cbx7* generates an altered chromatin structure that is susceptible to stochastic epigenetic or genetic secondary hits, which ultimately shape the disease phenotype. Identification of secondary genetic abnormalities would require additional assessment of the mutational status of subclones, which is beyond the scope of the present study.

The dynamic clonal behavior and conversion of leukemic phenotype that we observed in our mouse model show resemblance with patients presenting with leukemic relapse after treatment. First, if remissions in relapsed patients are treated similarly as the original leukemia, the response is often different (Leung et al., 2013; Patel et al., 2013; Verma et al., 2010). It seems likely that distinct leukemic clones exhibit a distinct response to therapeutic interventions, reinforcing the relevance to assess the functional consequences of clonal heterogeneity within leukemia. Second, lineage conversions in relapsed leukemia patients have been reported recurrently. A conversion from ALL to AML is most common, particularly in pediatric patients (Gagnon et al., 1989; Grammatico et al., 2013; Imataki et al., 2010; Rossi et al., 2012; Shivarov et al., 2009; Stass et al., 1984; van den Ancker et al., 2009). Conversions from AML to ALL occur less frequently, but have been reported for both children and adults (Bernstein et al.,

### Figure 5. Activation of a Dormant LCS Clone Result in the Appearance of an Undifferentiated Leukemia Subtype

(A) Experimental setup. Serial transplantation of bone marrow cells from mouse 4 with a T cell leukemia into three secondary recipients. Recipients developed either a T cell leukemia (4-1 and 4-2) or a leukemia with an undifferentiated (lineage-negative) phenotype (4-3). (B) Cell counts in blood, bone marrow, and spleen and spleen weight of all mice are indicated. Reference blood counts of control non-leukemic mice are indicated by the gray box (dashed line refers to the mean values). (C) Percentage of different donor-derived GFP<sup>+</sup> hematopoietic cell types in blood, bone marrow, and spleen of mouse 4 and its recipients. (D) The contribution of different clones to the blood, bone marrow and spleen of donor mouse 4 and the clonal composition of blood and spleen of recipients in sorted hematopoietic populations (GR1<sup>+</sup> granulocytes, CD3e<sup>+</sup> T cells, or immature LIN<sup>−</sup> cells) of indicated mice. The size of the pies reflects the percentage of cells in the indicated tissue. Different colors represent different clones and are indicated by different numbers. (E) Heat map summary and hierarchical clustering of gene expression analyses, illustrating differential expression of genes in unfractionated spleen cells from mouse 4-3 (with an undifferentiated leukemia) compared with spleen cells from mice 4-1 and 4-2 with T cell leukemias (also see Figure S5A).



**Figure 6. Activation of LCS Clones after Serial Transplantation**

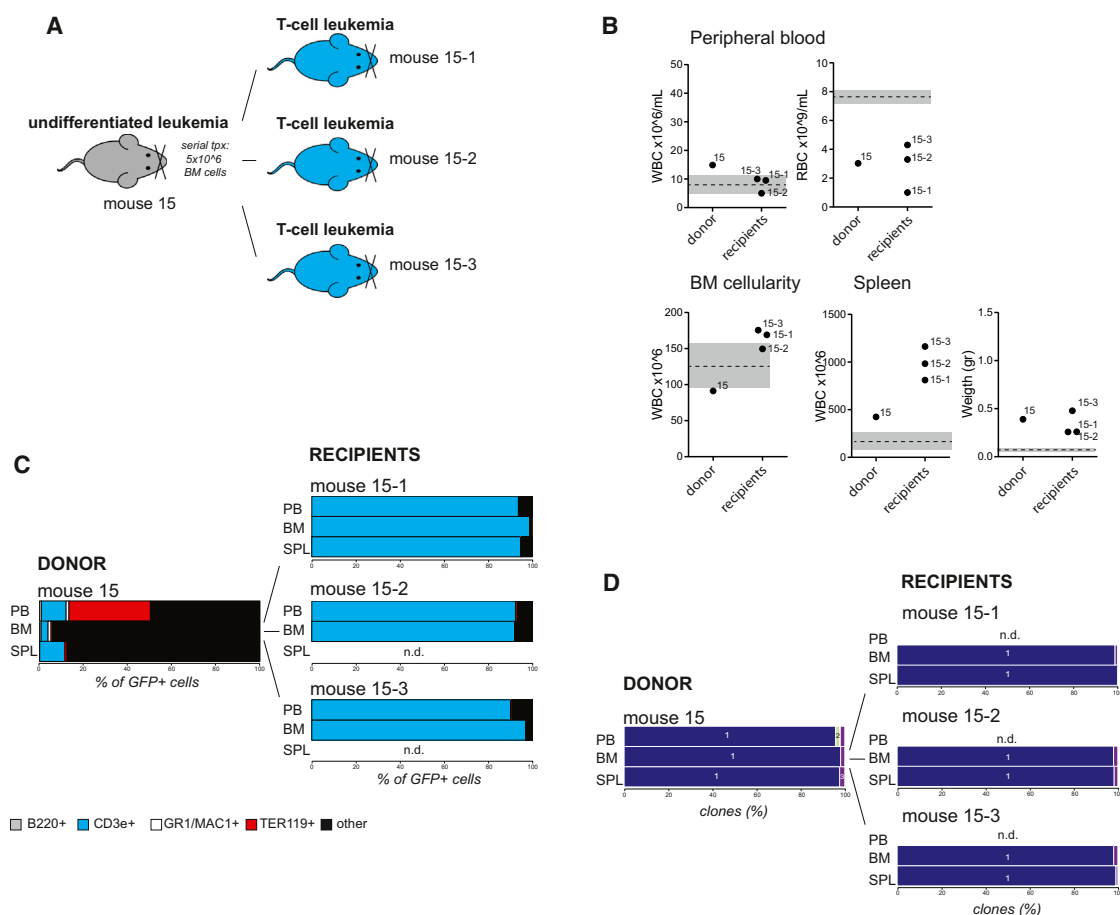
(A) Overview of the serial transplantation experiment in which bone marrow cells from mouse 5 with an oligoclonal T cell leukemia were serially transplanted into five secondary recipients. The majority of the recipients developed T cell leukemias (mice 5-1, 5-2, 5-3, 5-4). Recipient mouse 5-5 developed an erythroid leukemia.

(B) Cell counts in blood, bone marrow, and spleen and spleen weight of all mice are indicated. Reference blood counts of control non-leukemic mice are indicated by the gray box (dashed line refers to the mean values).

(C) Percentage of different donor-derived GFP+ hematopoietic cell types in blood, bone marrow, and spleen of donor mouse 5 and its recipients.

(D) The contribution of different clones to the blood, bone marrow, and spleen of mouse 5 and its five recipients. Different colors represent different clones and are indicated by different numbers.

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**Figure 7. Differentiation of Immature LSC Clones**

(A) Experimental setup. Bone marrow cells from a mouse with an immature, lineage-negative, leukemia (mouse 15) were serially transplanted into three recipients (mice 15-1, 15-2, and 15-3). All recipients developed T cell leukemia.

(B) Cell counts in blood, bone marrow, and spleen and spleen weight of all mice are indicated. Reference blood counts of control non-leukemic mice are indicated by the gray box (dashed line refers to the mean values).

(C) Percentage of different donor-derived GFP+ hematopoietic cell types in blood, bone marrow, and spleen of donor and its secondary and tertiary recipients.

(D) The contribution of different clones to the blood, bone marrow, and spleen of donor mouse 15 and its recipients. Different colors represent different clones and are indicated by different numbers.

1986; Boeckx et al., 2004; Dorantes-Acosta et al., 2009; Emami et al., 1983; Krawczuk-Rybak et al., 2003; Lounici et al., 2000; Marcus et al., 1985; Rossi et al., 2012). Case reports of other types of leukemic conversions, such as erythroid leukemia into ALL, have also been published (Park et al., 2011). In leukemic patients showing a relapse

of a different lineage (so-called lineage switch), leukemic clones often have a different morphology, cell size, amount of cytoplasm, presence of Auer rods, and new phenotypic lineage markers (Park et al., 2011; van den Ancker et al., 2009). These clinical data are highly reminiscent of our current findings.

(E) Heat map summary and hierarchical clustering of gene expression analyses, illustrating differential expression of genes in unfractionated spleen cells from mouse 4-3 (with an undifferentiated leukemia) compared with spleen cells from mice 4-1 and 4-2 with T cell leukemias (also see Figure S5A).

(F) Integration site analysis by iPCR using the restriction enzyme MluI. Colored triangles identify the corresponding barcodes and were confirmed by conventional PCR (data not shown). Gel electrophoresis of amplified integration sites showed signs of dominance of clone 2 in bone marrow cells of mouse 5, dominance of clone 3 in mouse 5-1 bone marrow cells and clones 1 and 3 in mouse 5-2. iPCR validated clone 2 as being dominant in mouse 5-4 and mouse 5-5, although these mice show different leukemia subtypes.





Overall, we conclude that the clonal makeup of leukemias can be more complex than often anticipated. Although the exact role of *CBX7* in human leukemias is currently unknown, we provide direct evidence for the quiescent nature of some LSCs and show lineage conversion at the clonal level. Leukemias can be oligoclonal, with clone-dependent variability in multiple biological traits. As not all clones are equal, therapies should focus on eliminating all clones with variable behaviors, including quiescent LSCs. As such, the finding of clonal heterogeneity at diagnosis argues in favor of adopting combination rather than single-agent sequential therapies with the goal to eradicate dominant as well as minor clones that may emerge at relapse. The barcoding approach that we advocate in the current study has been shown to be very powerful in delineating the behavior of normal HSCs (Gerrits et al., 2010; Verovskaya et al., 2013) and will also be of benefit to the study of preclinical tumor clonality models.

## EXPERIMENTAL PROCEDURES

Primary post-5FU bone marrow cells were isolated from C57BL/6.SJL (CD45.1) mice and transduced with a control or *Cbx7* barcode vector library as described previously (Gerrits et al., 2010);  $4.5\text{--}7.5 \times 10^6$  were transplanted into lethally irradiated (9.0 Gy) CD45.2 mice without prior GFP sorting. Analysis of the contribution to blood was performed at indicated time point, as described previously (Klaue et al., 2013). For secondary transplantations, 5 million whole bone marrow cells were transplanted into lethally irradiated recipients. Barcodes were recovered by extraction of genomic DNA (Gerrits et al., 2010), and individual samples for each deep sequencing run were amplified with assigned multiplexing primers as previously described (Verovskaya et al., 2013). In some cases, barcode data were validated by sanger sequencing and/or by inverse (iPCR).

For gene expression analysis, total RNA was isolated from bone marrow cells from selected mice using the RNeasy Mini Kit (QIAGEN). ss cDNA was generated and hybridized onto the Illumina MouseRef8 v.2 BeadChips according to the protocol from Illumina (ServiceXS). Primary expression analysis was performed using Illumina's Genomestudio v.2011.1 software with default settings. Data were normalized, and a differentially expressed analysis between erythroid and T cell leukemic samples was performed using limma *eBayes*. Two publically available data sets were used to compare between gene expression profiles of our leukemic cells and normal gene expression profiles (GSE6506 [Chambers et al., 2007]: LTHSCs, monocytes, B cells, natural killer cells, granulocytes, erythroid cells, naive CD4+ T cells, naive CD8+ T cells; GSE18669 [Weishaupt et al., 2010]: HSCs, MPPs, PreMegE, CD4+ T cells). We extracted genes that are specifically highest expressed in normal hematopoietic cell subsets, and these lists were then compared with our data sets (Python SciPy, hypergeometric test).

See Supplemental Experimental Procedures for a more detailed description of methods used in this study.

## ACCESSION NUMBERS

Normalized and raw gene expression data is available at the Gene Expression Omnibus (GEO) under GSE56820.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and three tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2014.10.012>.

## AUTHOR CONTRIBUTIONS

K.K., L.V.B., and G.d.H. initiated research and developed the concept of the paper. K.K. designed the experiments with help from L.V.B., and G.d.H.; K.K. performed experiments with contributions from M.J.C.B. and E.W., M.R., and A.D.A. contributed to experiments involving animal caretaking. M.V.G. assisted in optimization of IPCR. E.Z. performed bioinformatics analyses with contributions from L.V.B. and K.K.; K.K. and L.V.B. analyzed and interpreted data, and K.K. wrote the manuscript with contributions from L.V.B. and G.d.H.

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